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14. ABSTRACT

The p53 transactivation domain 1 (TAD1) plays a critical role in inducing p53 mediated cell-cycle arrest and apoptosis in response to acute DNA damage caused by irradiation. During radiation therapy of cancers, this p53-induced apoptosis triggers various deleterious pathological side effects in normal tissues. Interestingly, recent studies from our laboratory have demonstrated that p53 TAD1 is completely dispensable for tumor suppression in diverse mouse cancer models. We hypothesize that specific inhibition of p53 TAD1 should ameliorate the p53-associated pathologies occurring in response to acute DNA damage, while keeping p53-mediated tumor suppression intact, thus allowing improvement in the therapeutic index of radiation therapy in cancer. Importantly, because the majority of cancers, such as advanced prostate cancers, have inactivated the p53 pathway, such inhibitors should not compromise the efficacy of treating tumors. We propose to perform high-throughput chemical library screens to identify specific inhibitor of p53 TAD1 that may be administered as adjuvants of chemotherapy and radiotherapy in the context of prostate cancer.

15. SUBJECT TERMS
p53 inhibitor, p53 transactivation mutants

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Introduction

The p53 protein plays a pivotal role in suppressing tumorigenesis, as evidenced by its inactivation in over half of human cancers. Through its ability to function as a transcriptional regulator, p53 has the capacity to respond to a variety of genotoxic stress signals and induce cell-cycle arrest, apoptosis and cellular senescence to curb neoplastic growth (*1*). Aside from its beneficial tumor suppressive capability, p53 is also a critical mediator of DNA damage signals and this property provokes it to induce deleterious, pathological side effects associated with genotoxic stress-inducing radio- and chemotherapies, particularly in the radiosensitive tissues. The active role of p53 in provoking the detrimental side effects of cancer therapy has opened up the possibility of developing specific p53 inhibitors that can be used as radiotherapy and chemotherapy adjuvants to reduce these side effects. It would be ideal to identify p53 inhibitors that could suppress p53-associated pathologies without perturbing p53 tumor suppressor function. Previously, we established that p53 transactivation domain 1 (TAD1) is critical for inducing cell cycle arrest and apoptosis in response to acute DNA damage but is dispensable for p53-mediated tumor suppression in various mouse cancer models (*2, 3*). Based on these observations, we hypothesize that inhibition of p53 TAD1 will selectively inhibit p53-dependent, radiation-induced cell death and subsequent normal tissue damage during radiation and chemotherapy of p53-negative cancers. Towards this end, we propose to perform a high-throughput chemical library screen to identify p53 TAD1 inhibitors by generating and using a mouse embryonic fibroblast reporter system that expresses dual color luciferases under the control of p53 TAD1-dependent and independent target gene regulatory elements.

Body

Specific Aim 1: To generate mouse embryonic fibroblasts (MEFs) stably expressing p53-inducible fluorescence reporters for screening

Our major goal is to identify p53 transactivation domain 1-specific inhibitors by generating and subjecting reporter MEFs to a chemical library screen. Towards this goal, originally, we proposed to use lentiviral plasmids to construct wild-type and p53 TAD mutant MEFs stably expressing dual-fluorescence reporter system, in which the p53 responsive regulatory sequences of a p53 TAD1-dependent gene, *Cdkn1a* (or *p21*), and that of a TAD1-independent gene, *Bax*, are fused to two different fluorescent reporters. After consultations with several researchers who have extensive experience with using reporter cell lines for chemical library screening, we have adopted the following key changes to improve our screening strategy.

1) Using *Crip2* instead of *Bax* as a p53 TAD1-independently expressed gene.

To identify p53 TAD1-specific inhibitors, we had proposed to utilize the p53 responsive regulatory sequences from *Bax*, a pro-apoptotic member of the Bcl-2 protein family, which is expressed independently of p53 TAD1 function as determined by Northern blotting and qRT-PCR analysis of an allelic series of p53 transactivation mutants in DNA-damage-treated MEFs. More recently, through qRT-PCR analysis of a larger panel of p53-target genes, we were able to identify another such gene, *Cysteine-rich intestinal protein 2* (*Crip2)*, whose expression is unperturbed upon mutation of p53 TAD1 in MEFs. In this analysis, consistent with our previous results, the basal expression of *p21* as well as its induction upon acute DNA damage was significantly reduced (5-6 fold) in p53 TAD1 ($p53^{25,26}$) but not TAD2 mutant ($p53^{53,54}$) MEFs relative to wild-type MEFs, suggesting that *p21* expression is solely reliant on p53 TAD1 (Figure 1A). The substantial difference in *p21* expression between the p53 wild-type and p53 TAD1 mutant MEFs makes it an optimal choice for detecting measurable changes in a bioluminescence based high-throughput screening assay. In contrast, the basal expression of *Crip2* did not change significantly in either the p53 TAD1 or TAD2 single mutant MEFs but was significantly reduced upon mutation of both p53 TADs ($p53^{25,26,53,54}$), suggesting that either of the two p53 TADs can stimulate *Crip2* expression and that it is an optimal reporter of p53 TAD1-independent expression (Figure 1B). Based on these data, we plan to pursue the chemical library screens under basal expression conditions since we observed notable differences between *p21* and *Crip2* expression patterns only under basal conditions but not under DNA damage inductions. Further qRT-PCR analysis of MEFs and lung tumor cells revealed that p53-dependent regulation of *Crip2* at the basal level is more robust than that of *Bax* (data not shown). Thus, *Crip2* appears to be a more appropriate choice for p53 TAD1-independent category gene since it is expected to provide better reporter gene basal expression than *Bax*.

2) Using *Arf* null immortalized MEFs instead of wild-type MEFs for generating reporter cell lines

Previously, we proposed to construct reporter cell lines in the p53 wild-type and p53 TAD mutant MEF genetic backgrounds. A major limitation with using wild-type MEFs is that they undergo replicative senescence within a relatively short span of time in cell culture. Hence, after successful introduction of the reporter genes, wild-type reporter cells can be used for screening assays for a very limited period of time. This becomes a major issue when trying to reproduce results of a screening assay in a given wild-type cell line. Hence, we now plan to use p53 proficient *Arf* null immortalized MEFs as the genetic background for constructing the reporter lines. Unlike their wild-type counterparts, the *Arf* null MEFs do not undergo replicative senescence and will therefore provide us with a virtually inexhaustible source of cells to conduct our studies. We first intercrossed

Arf heterozygous mice and generated *Arf* null MEFs. Then, since $p19^{Arf}$ is a well-known upstream regulator of the p53 pathway, we next determined whether the p53-DNA damage response is intact in these cells. We induced DNA damage in *Arf* null MEFs by doxorubicin treatment and compared the expression profile of several p53 target genes to that of littermate wild-type control MEFs. We observed that p53 target gene induction by DNA damage in *Arf* null MEFs was indistinguishable from that observed in wild-type MEFs, suggesting that these cells possess intact p53 transcriptional activity in response to DNA damage and are therefore suitable for use in our screening assays (**Figure 2A**).

We have also assessed the feasibility of using human fibroblasts to identify specific inhibitors of the human p53 TAD1. For this purpose, we obtained a hTERT-immortalized human fibroblast cell line and have tested p53 transcriptional responses to acute DNA damage treatments. Most of canonical p53 target genes were upregulated after doxorubicin treatment, suggesting that this system can also be used to generate cell lines with p53 reporters (**Figure 2B**).

3) Using click beetle-derived luciferase reporters in addition to fluorescent reporters for high-throughput screening.

While fluorescent reporters are considered to be a suitable choice for high-throughout screenings, they have certain limitations. In general, fluorescent assays tend to have much higher backgrounds, leading to lower relative signals. Compounds that inhibit fluorescence or interfering fluorophores within the cells can limit fluorescence assays. Also, analysis of fluorescent signals in a microtiter plate is a time consuming process and presents the problem of introducing a time of incubation variable to the cells undergoing the screening process. Some of the drawbacks of fluorescent reporter based assays can be alleviated by the use of Chroma-Glo™ dual color luciferase reporter system generated by Promega. This system is designed to generate click beetle-derived red and green luminescence from a single sample upon addition of a single reagent and permits each reporter to be measured independently and simultaneously, making it suitable for use in an automated high-throughput screening assay.

4) Generation of dual reporter cell line using gene editing instead of lentiviral vectors

Previously, we proposed to use lentiviral vector systems to generate our reporter cell lines. However, due to the well-established variable integration of lentiviral vectors into different sites, often resulting in sub-optimal expression of reporters, we will harness multiple site-directed genome editing approaches to directly modify the *p21* and *Crip2* loci such that the expression of the dual-reporter system is governed by the endogenous regulatory elements of these genes.

Recently, the genome-editing field has further expanded with the exciting

development of Tal-effector and CRISPR guided nucleases. Transcription activator-like effector nucleases (TALENs) are engineered proteins that enable precise alterations at endogenous genomic loci by stimulating specific DNA double-strand breaks (*4, 5*). These are composed of a specifically engineered DNA binding domain fused to the FokI endonuclease domain. These are designed as a pair that binds to contiguous genomic sites flanking a target site leading to dimerization of the FokI domain and resulting in a highly specific DNA double-strand break. These double stranded breaks can be repaired by either the error prone non-homologous end-joining pathway (NHEJ) or by homology-directed repair with a homologous DNA donor template, which can be exogenously introduced to incorporate precise alterations.

Site-directed genome editing can also be achieved by the recently developed CRISPR-Cas9 system. CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats) is widely believed to be the most efficient method to engineer mammalian genomes. CRISPR RNAs (crRNA) that hybridize to a specific target DNA can be utilized to guide a double strand break-inducing Cas9 nuclease at a locus of interest (*6*). For our studies, we will employ the two-component pX330 vector system developed by the Zheng lab at MIT, in which the crRNA and Cas9 expression are constitutively driven by the mammalian U6 and Chicken β-actin short promoter (CBh) respectively (*7*).

To generate reporter cells that express red luciferase protein from the endogenous *p21* genomic promoter, we will target a promoterless red luciferase in-frame to the endogenous p21 ATG start site. In *Crip2*, the p53-binding site is in the first intron as determined by our ChIP-seq analysis (*8*). With *Crip2*, we will target a promoterless green luciferase in-frame to the endogenous *Crip2* Exon 2 to preserve the structural integrity of the p53 response element (which resides in the first intron) at this locus. To achieve this targeting, we have generated a series of donor constructs that contain approximately 1 kilobase arms of homology 5' and 3' of the TALEN / CRISPR cut sites. In between the homology arms, we have included a promoterless luciferase and an Ubiquitin C promoter-driven Puromycin expression cassette which, upon successful homologous recombination, would be stably integrated into the locus (**Figure 3A**). To create a dual-fluorescent reporter that expresses red luciferase from the endogenous *p21* locus and green luciferase from the endogenous *Crip2* locus, we will use the *Crip2* TALENs/CRISPRs to target the *Crip2*-in-frame-green luciferase vector to the *Crip2* locus in a previously targeted *p21*-red luciferase clone. As an alternative fluorescent reporter based screening strategy, we will also generate a dual-fluorescent reporter cell line that expresses GFP under the control of *p21* promoter and TdTomato under the control of *Crip2* promoter (**Figure 3B**). To determine the efficiency of targeted integration, we will employ the single molecule, real time (SMRT) sequencing methodology (*9*).

Thus far, we have successfully generated 4 pairs of TALENs and 3 CRISPRs for each of the *p21* and *Crip2* loci and are currently determining their cutting

efficiencies using standard nuclease surveyor assays. We have also completed the construction of all the desired targeting vectors. We have optimized introduction of the *p21* and *Crip2* TALENs and CRISPRs into the *Arf* null immortalized MEFs through nucleofections using the Amaxa nucleofector device. By using the Amaxa proprietary program #T-20, we were able to obtain a more than adequate >85% nucleofection efficiency with the *p21* TALENs (data not shown). We are poised to introduce the targeting vectors imminently. Dr. Matthew Porteus, an expert in this area at Stanford University, is actively advising us on all steps of this protocol.

Specific Aim 2: To conduct chemical library screen for compounds inhibiting *p21* but not *Bax* fluorescence reporter activity in wild-type MEFs upon DNA damage treatment

As described above, we have made a few technical changes to this aim. First, we will use *Crip2* gene reporters instead of *Bax* as p53 TAD1-independent reporters. Second, we will use *Arf* null MEFs instead of wild-type MEFs to construct the reporter cell lines. And lastly, in addition to fluorescent reporters, we will generate luciferase reporter cell lines for our high-throughput screening. The Stanford High-Throughput Bioscience Center (HTBS) has the instrumentation for both dual color luciferase as well as dual fluorescent reporter system based high-throughput screenings.

Specific Aim 3: To confirm that the candidate compound(s) inhibits p53-dependent acute DNA-damage responses and improves prostate cancer treatment in mouse models

No changes.

Key Research Accomplishments

1) Tested a panel of p53 target genes to identify several p53 TAD1-dependent and TAD1-independent genes. Further validated *Cdkn1a (p21)* and *Crip2* as p53 TAD 1-dependent and -independent target genes, respectively, through luciferase reporter assays and qRT-PCR analysis in p53 TAD mutant MEF lines.

2) Generated key reagents required to prepare reporter cell lines that will be subjected to a chemical library screen
   a) Construction of plasmid vectors that express *p21* and *Crip2* genomic locus-specific TALEN-FokI and CRISPR-Cas9 endonucleases.
   b) Construction of an array of targeting vectors that introduce either fluorescent or click beetle luciferase reporters at the *p21* and *Crip2* loci
   c) Preparation of Arf null mouse embryonic fibroblast cells for the targeting of the reporters

3) Optimized a nucleofection protocol to introduce plasmids expressing CRISPRs and TALENs into immortalized MEFs

Reportable Outcomes

So far, we have taken some key steps towards the development of reporter cell lines for a chemical library screening that we propose to perform. We believe that these reporter cell lines will turn out to be an invaluable resource for studying p53 transactivation function in general.
This work was presented in poster presentation at the annual Stanford University Department of Genetics retreat 2013 that was held at Monterey, CA on 9/18/13 and was well received.

Conclusions

Our laboratory has provided the first identification of a mechanism distinguishing p53 action in acute DNA damage responses and in tumor suppression, which we leverage here to identify compounds that could be used to mitigate side effects of prostate cancer therapies without causing risk of additional cancer development. Identification of TAD1 inhibitors could allow protection from radiation-induced toxicity to normal tissue, thereby allowing enhanced doses of radiation to be administered and enhancing prostate cancer treatment. The experiments described above will help perform a screen to identify such TAD1 inhibitors.

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Appendices

Figure 1

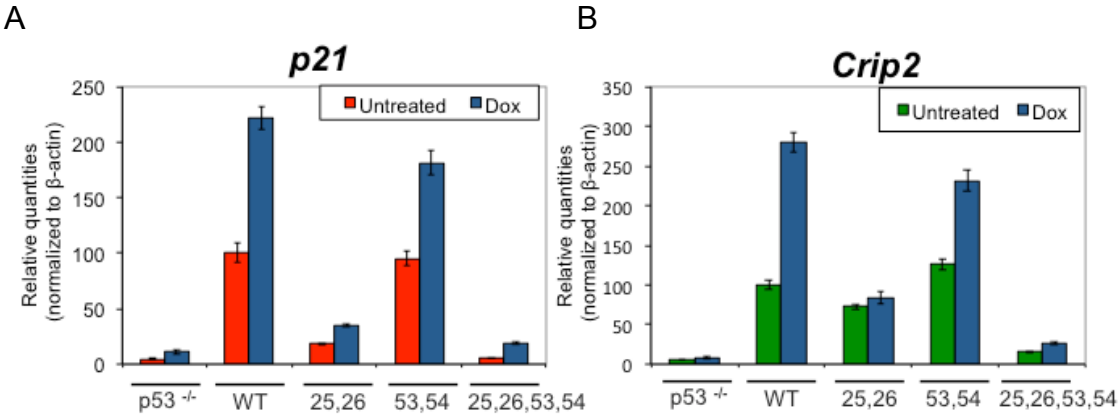

Fig.1 qRT-PCR analysis of p53 target genes, *p21* and *Crip2*, in DNA damage-treated MEFs expressing p53 transactivation mutants. p53 transactivation mutant-expressing MEF lines were treated with 0.2 μg/ml of DNA damaging agent, doxorubicin, for 8 hours. Graphs indicate averages +/- standard deviation of quantities normalized first to β-actin and then to wild-type untreated samples.

Figure 2

A

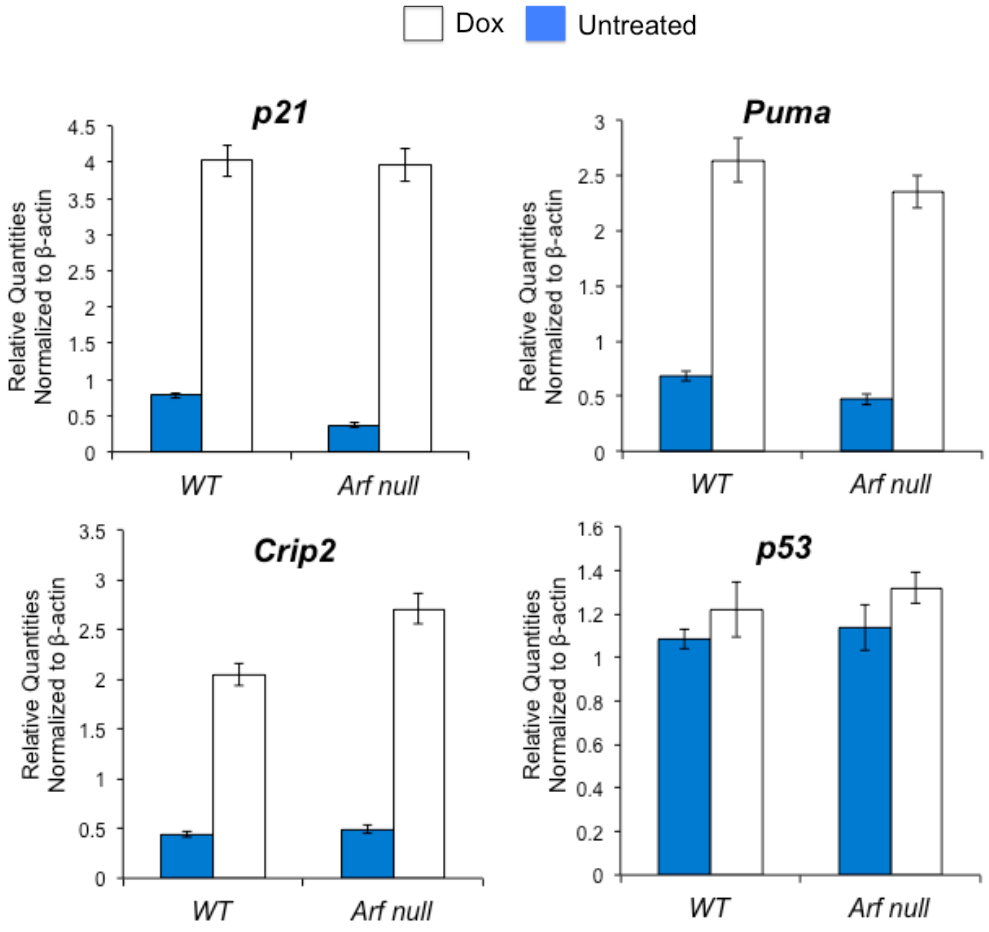

Fig.2A qRT-PCR analysis of *p53* and its target genes *p21*, *Puma*, and *Crip2* in DNA damage-treated *Arf* null and littermate wild-type (WT) control MEFs. Cells were treated with 0.2 µg/ml of DNA damaging agent, doxorubicin, for 8 hours. Graphs indicate averages +/- standard deviation of quantities normalized to β-actin.

Figure 2 contd.

B

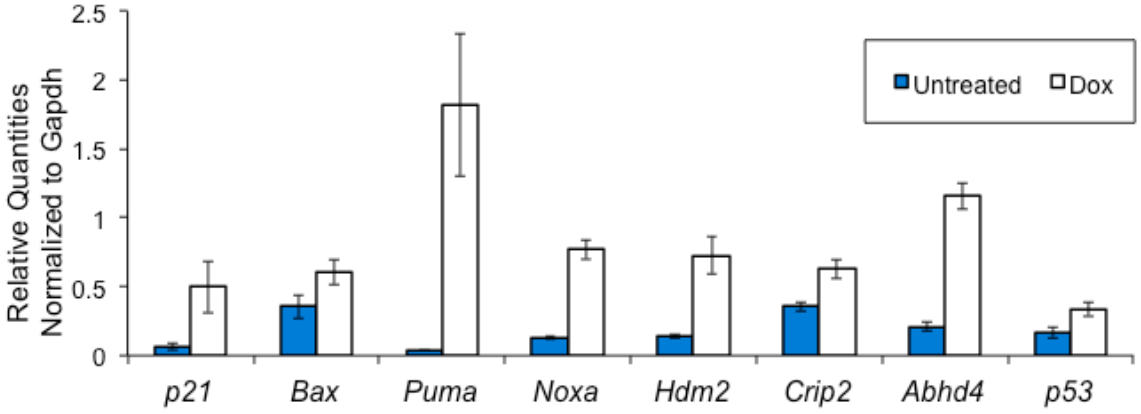

Fig. 2B. qRT-PCR analysis of p53 target genes in human fibroblasts treated with DNA damaging agent doxorubicin. Bar graphs indicate averages +/- standard deviation of quantities normalized to *Gapdh*.

Figure 3.

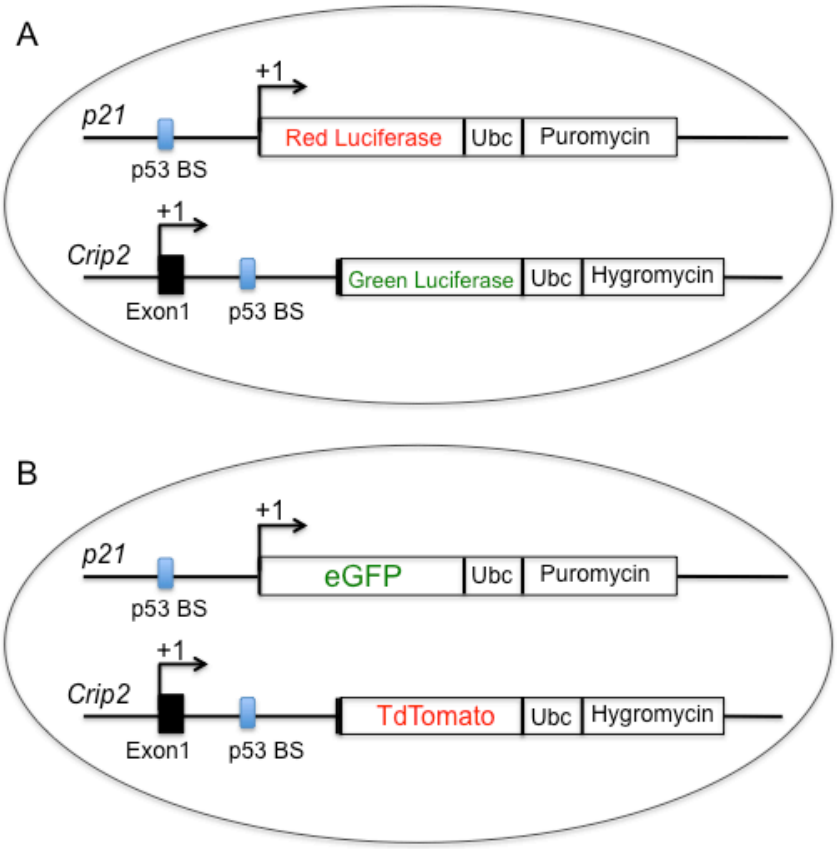

Fig. 3. Schematic view of the reporter cell lines that express click beetle-derived red and green luciferases (A) or fluorescent reporters (B) under the control of *p21* and *Crip2* regulatory sequences, respectively.